Environmental Health Perspectives
Vol. 31, pp. 141-149, 1979

Analysis of Aneuploidy in First-Cleavage Mouse Embryos Fertilized *in Vitro* and *in Vivo*

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First-cleavage mouse embryos, fertilized *in vitro* and *in vivo*, provide ideal material for chromosomal analysis. With the appropriate incubation in a mitotic inhibitor, syngamy is prevented and the sperm- and egg-derived chromosomes remain as separate clusters. Because the latter chromosomes undergo condensation sooner than those from the spermatozoon, the parental source of chromosome sets can be identified even without a marker chromosome. Thus these embryos can be analyzed both for the primary incidence and the parental source of a number of chromosomal anomalies, including aneuploidy. By using fertilization *in vitro* to obtain the embryos, the synchrony of fertilization and nuclear development is such that 80% or more of the chromosomal preparations are suitable for analysis, compared with about 50% for embryos fertilized *in vivo*.

The detection of aneuploidy in mammalian embryos is dependent, to a certain extent, on the age of the embryos, because the proportion of fetuses which are aneuploid decreases as pregnancy advances (*1*). Preimplantation stages are easily recovered, and the incidence of aneuploidy detected in such embryos more closely reflects the primary incidence. In any of these stages, however, the source of aneuploidy, i.e., whether of maternal or paternal origin, is not unequivocal due to the mingling of the two sets of chromosomes at syngamy, prior to first cleavage, which restores the diploid state. If analysis is carried out before syngamy, the incidence of aneuploidy and indeed other chromosomal anomalies can be assessed in both maternal and paternal contributions. Using the mouse, we have developed a system which permits such analysis in a high proportion of first-cleavage embryos examined. The unique feature of the system is the use of fertilization *in vitro* to obtain the embryos for analysis. Embryos fertilized *in vivo* have also proved suitable, although the former yield a higher proportion of scoreable preparations because of greater synchrony of fertilization *in vitro* and subsequent nuclear development. With the appropriate application of a mitotic inhibitor *in vitro*, the egg- and sperm-derived chromosomes do not mingle, but rather remain in discrete clusters, thus making possible the assessment of both parental contributions to the zygote, and hence the primary incidence as well as source of anomalies.

Although we have not examined the effects of mutagens on the incidence of aneuploidy, our system could be used for this purpose quite easily. We therefore describe the basic techniques and then discuss results we have obtained in experiments where factors other than mutagens, e.g., genetic ones, were being examined for their effect on gamete interactions and subsequent polyploidy and aneuploidy.

Materials and Methods

Mice

Mice from a large number of mouse strains have been used as both egg and sperm donors, including the inbred C57BL/10, CBA, DBA/2, CBA/H-T6, (C57BL/10 × CBA) F_1 and the outbred TO. Strains differ in the number of eggs shed in response to exogenous hormones and in the sperm quality. For routine work, gametes from TO and (C57BL/10 ×

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CBA) F_1 mice have provided consistently high levels of fertility which is of particular importance for fertilization *in vitro*.

All female mice were induced to superovulate by the administration of exogenous hormones. Superovulation has three advantages: (1) there is greater yield of eggs/mouse [20-40/female, depending on strain of mouse; (*2*)], (2) there is a synchrony of ovulation, and (3) the eggs obtained will be recently ovulated. The last is an important consideration for fertilization *in vitro*, since aged eggs are less fertile and more susceptible to parthenogenetic (spontaneous) development (*3*). Adult (2-4 month old) virgin females were injected intraperitoneally (IP) with 7.5 I.U. pregnant mare serum (PMS: Gestyl, Organon) and approximately 48 hr later with 5.0 I.U. human chorionic gonadotrophin (hCG: Pregnyl, Organon), also given I.P. Depending on the strain of mouse used, ovulation will be complete 12-14 hr after injection of hCG (*2*).

Adult (2-4 month old) males are used either for mating *in vivo* or as sperm donors for fertilization *in vitro*. All mice are maintained on a light cycle of 14 hr light and 10 hr dark.

Fertilization *in vitro*

The medium used for all manipulations of gametes is a simple salt solution based on Tyrode's solution, plus pyruvate, lactate, and bovine serum albumin (BSA). For fertilization, the medium contains 32 mg BSA (Armour)/ml and for culture, 4 mg BSA/ml (*4*).

Sperm suspensions are prepared by placing two cauda epididymides into a plastic culture dish (30 mm) containing 1 ml of fertilization medium and overlaid with paraffin oil (*4*). Spermatozoa are forced out by exerting pressure with two pairs of watchmaker's forceps, allowed to disperse for 20 min, and then diluted to a concentration of approximately 2×10^6 spermatozoa/ml, although this can be varied and still permit high levels of fertilization (*4, 5*). The diluted suspension is transferred to a culture dish containing only paraffin oil.

Females are killed 13-14 hr after hCG, i.e. upon completion of ovulation, and the oviducts are removed; unfertilized eggs are then released from the oviducts directly into the diluted sperm suspensions. The dishes are placed in an anaerobic culture jar which is gassed with either 5% CO_2 in air or 5% CO_2, 5% O_2, 90% N_2; alternatively, a CO_2 incubator, if available, can be used.

After incubation for 6 hr at 37°C, the eggs are removed from the sperm suspensions, washed once in culture medium and transferred to a small droplet of culture medium, containing vinblastine sulfate, 10^{-5} *mM* (Velbe, Lilly), under oil and cultured overnight. The following morning chromosome preparations are made. In our experience, eggs lacking a second polar body at the end of the 6 hr incubation are almost always unfertilized and these are usually discarded; thus, relatively rare digynic embryos may be missed.

Chromosome Preparations

The methods used are essentially those of Tarkowski (*6*), with a short exposure of the embryos to a hyptonic citrate solution followed by fixation with methanol:acetic acid (*7*). Preparations are stained with 1% aqueous toluidine blue and after examination, slides can be washed, dried and stored for later re-examination if desired.

Difficulties in interpretation are encountered infrequently and the majority of all counts are confident. If a chromosome spread suggests excessive scatter with accompanying loss of chromosomes, it is rejected. The fact that each embryo consists of a single cell with discrete pronuclei reduces the chance of overlap that is frequently encountered with later, multicellular stages. There is the added advantage that all the embryos, assuming normal development, will enter mitosis for the first-cleavage during the period of incubation in the mitotic inhibitor.

Fertilization *in vivo*

Females are injected with hCG 13 hr prior to the estimated time of sponteneous ovulation and then paired overnight with males. If vaginal plugs, indicating mating, are found the following morning, the females are killed in the late afternoon; the fertilized, 1-cell embryos are recovered, washed, and cultured overnight in medium containing Velbe, then processed as detailed above. We have found that if these embryos are put into the mitotic inhibitor too early, the chromosome condensation is frequently stopped at late prophase and the resulting preparations cannot be analyzed for aneuploidy; for this reason, we stipulate late afternoon as the time for embryo recovery.

Equipment Needed for Fertilization *in vitro* And Embryo Culture

Equipment required is an incubator, anaerobic culture jar, dissecting microscope, gas mixture (5% CO_2 in air or 5% CO_2, 5% O_2, 90% N_2), water bath, gas washing bottle, and warming tray.

Results

The overnight incubation of recently fertilized mouse embryos in a mitotic inhibitor effectively pre-

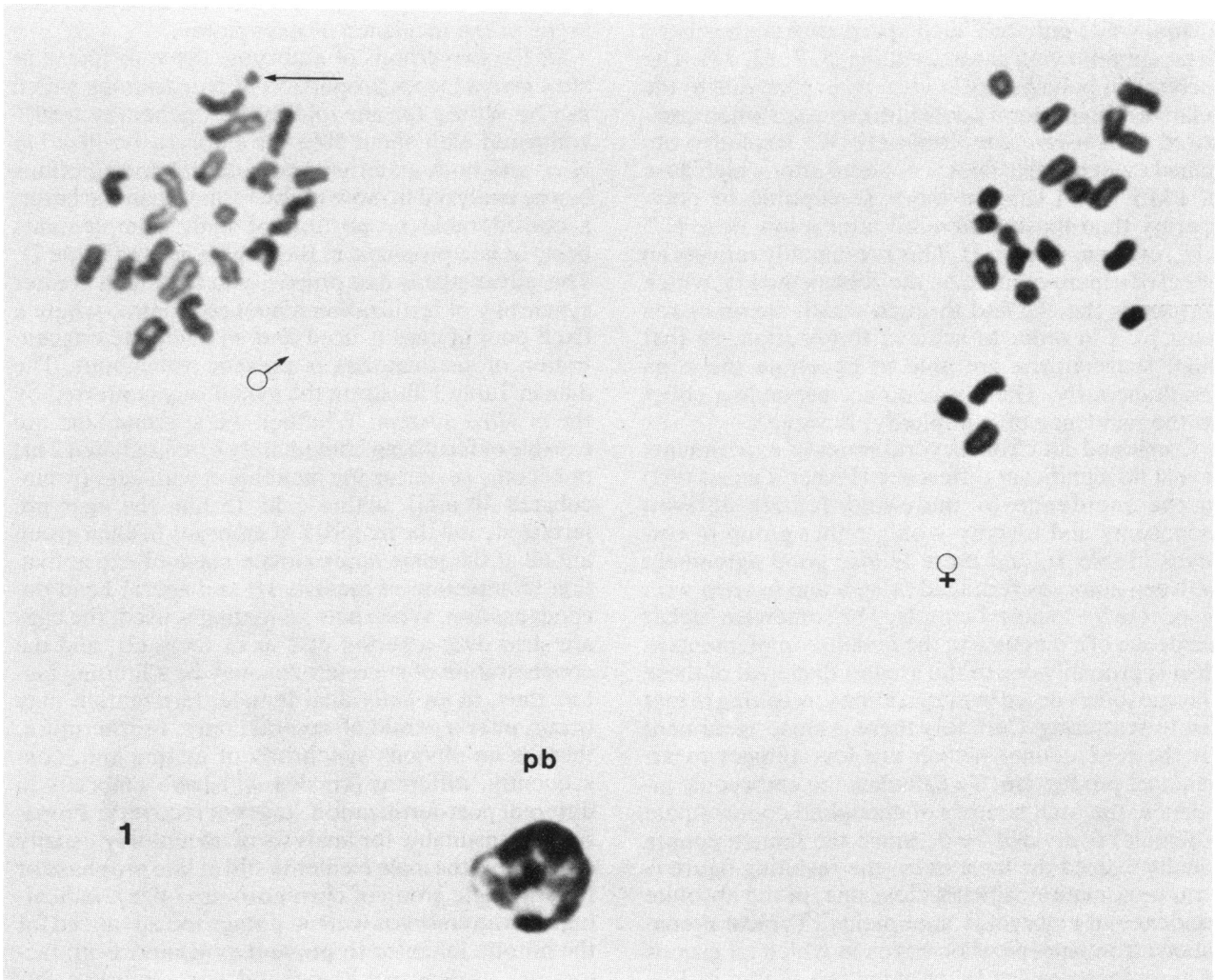

FIGURE 1. Diploid first-cleavage mouse embryo; sperm donor was a CBA/T6 male and the T6 marker chromosome is denoted by the arrow: (♂) male-derived complement; (♀) = female-derived complement; pb = second polar body.

vents syngamy, and the egg- and sperm-derived chromosomes are visible as distinct groups. Initially we used CBA/H-T6 males in order to have a distinguishing marker in the chromosome set contributed by the spermatozoon (Fig. 1), but it became evident that sufficient differences existed between maternal and paternal contributions to make a marker unnecessary. The maternally derived chromosomes exhibit a greater degree of condensation and thus stain more intensely and are generally more widely dispersed than are the paternally-derived ones. These features have in fact been noted by other workers (*8, 9*) and used in mutagen studies (*10, 11*). From a single preparation we can determine whether one or more spermatozoa have fertilized the egg, and in most embryos the female complement can be assessed for aneuploidy, although comparable analysis of the male complement is sometimes made difficult because these chromosomes, condensing later as they do, may still be in late prophase.

The most notable difference we have been able to detect using the *in vivo* and *in vitro* systems just described is the significantly higher incidence of polyspermy, primarily dispermy, in first-cleavage embryos fertilized *in vitro* when compared with ones fertilized *in vivo*. In a typical series, 64/452 (14.2%) embryos fertilized *in vitro* were polyspermic, 60 being dispermic triploids and four being trispermic tetraploids, compared with only 4 dispermic triploids out of 228 (1.8%) embryos fertilized *in vivo* (*7*). Another interesting result has been the observation that about 1% of the fertilizing spermatozoa in the *in*

vitro group have had diploid chromosome complements, while only one such spermatozoon has been detected following natural mating (*5, 7, 12, 13*). The increase in polyspermy *in vitro* is, in part, due to the relatively high sperm concentration used when compared with *in vivo* conditions (*5*). We have also obtained evidence that eggs recovered after a high dose of PMS (7.5 I.U.) are more susceptible to polyspermy than those recovered after a low dose (1.5 I.U.) of the hormone (*7*). This presumably reflects an effect of superovulation on the zona pellucida, which surrounds the egg and through which spermatozoa must pass in order to achieve fertilization, so that more spermatozoa are able to penetrate the zona simultaneously. There was no corresponding effect on the incidence of aneuploidy, however.

Combined data from several series of experiments reveal no significant difference (Fisher's exact test) in the incidence of male-and female-derived monosomy and trisomy within either group of embryos (Table 1), and there is also good agreement between embryos fertilized *in vivo* and *in vitro* with respect to $b+1$ and $n-1$ counts. The somewhat higher incidence of $n-1$ counts in the female complements *in vitro* is probably due to the greater dispersal of these chromosomes noted in preparations, resulting in loss due to scattering. Certainly there is close agreement for the n+1 counts, which are less subject to artefactual production. To calculate the embryonic incidence, the total number of aneuploid counts (male + female) is divided by 2. Since the female counts usually exceed the male ones, the resulting figure is an approximation, albeit a close one, of the absolute incidence of embryonic aneuploidy. Typical preparations from aneuploid embryos in which all groups of chromosomes can be counted are shown in Figures 2-4.

In one experimental series, five different strains of mice were used as egg donors and TO males were used as sperm donors (*13*). Within the *in vivo* and *in vitro* groups, no significant differences were found between male- and female-derived aneuploidy, and these data have been combined to give an embryonic incidence (Table 2). Furthermore, there were no significant differences between the two groups of embryos in the incidence of aneuploidy.

Of the two groups of embryos, those fertilized *in vitro* give a higher proportion of preparations which can be scored for aneuploidy i.e., generally >80% compared with about 50% for embryos fertilized *in vivo,* and both maternal and paternal contributions can be analyzed in more of the former than the latter, a considerable proportion of male complements being at late prophase in the *in vivo* group (Table 2). This advantage is due primarily to the much greater synchrony of fertilization achieved *in vitro,* where a fixed pool of eggs is used and an adequate concentration of spermatozoa is present throughout. The data in Table 3 illustrate the synchrony conferred by the *in vitro* system. Whether the spermatozoa are capable of fertilizing immediately (preincubated 2 hr) or become so during the incubation with eggs (preincubated 30 min), within 1 hr 15 min the eggs are fertilized, and the majority of embryos in each group are all at the same approximate stage of egg activation (completion of meiosis II) and sperm head decondensation. When natural mating is used, the eggs are shed over a period of 2 hr or more (*2*), and the concentration of spermatozoa may be a limiting factor; thus, in an individual female, fertilization may occur, over a period of several hours. Furthermore, there is no obvious synchrony of mating and, consequently, different females will have embryos in different post-fertilization stages at recovery. Preparations unsuitable for analysis of aneuploidy usually either have the male elements still at late prophase or have a single group of chromosomes (40±), indicating that the embryos were at a stage too advanced for the mitotic inhibitor to prevent syngamy. Both factors are particularly associated with embryos fertilized *in vivo* because of the asynchronous mating and fertilization discussed above.

In only one experimental series have we observed a significant difference in aneuploidy attributable to one or other parental source. In comparing the incidence of aneuploidy in eggs obtained from young virgin and old parous females, a significantly higher incidence of trisomy was noted in the eggs from old

Table 1. Combined data on the incidence of aneuploidy in first-cleavage mouse embryos fertilized *in vivo* and *in vitro*: monosomy, trisomy, and total aneuploidy.[a]

		Male-derived complement (%)	Female-derived complement	Embryonic incidence
$n+1$	*In vivo*	2/ 744 (0.27)	4/ 935 (0.43)	6/ 840 (0.71)
	In vitro	8/1880 (0.43)	9/1925 (0.47)	17/1903 (0.89)
$n-1$	*In vivo*	2/ 744 (0.27)	4/ 935 (0.43)	6/ 840 (0.71)
	In vitro	4/1880 (0.21)	19/1925 (0.99)	23/1903 (1.21)

[a]Total embryonic incidence ($2n\pm1$) *in vivo,* 12/ 840 (1.43); *in vitro,* 40/1903 (2.10).

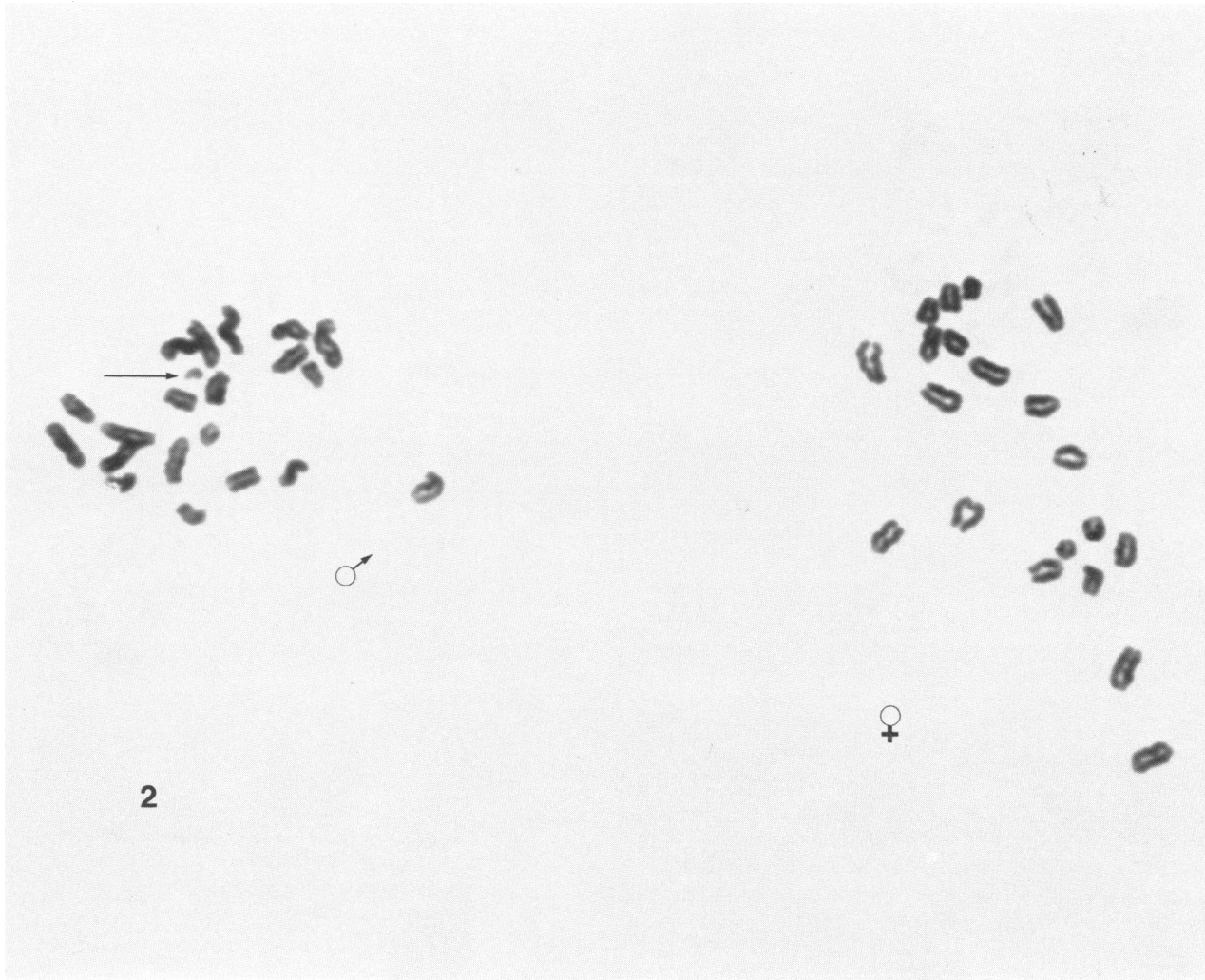

FIGURE 2. Trisomic first-cleavage mouse embryo. Sperm donor was a CBA/H-T6 male and the male-derived complement has 21 chromosomes. From Maudlin and Fraser (*13*) with permission.

Table 2. Incidence of aneuploidy in first-cleavage mouse embryos derived from eggs of five strains of females and fertilized *in vitro* and *in vivo* with TO spermatozoa.

		No. of embryos (%)			
Egg donor	Fertilization	Scored	Monosomic	Trisomic	Total aneuploid
DBA/2	*In vitro*	174	3 (1.7)	1 (0.6)	4 (2.3)
	In vivo	20	0 (0)	0 (0)	0 (0)
CBA	*In vitro*	90	1 (1.1)	0 (0)	1 (1.1)
	In vivo	29	0 (0)	0 (0)	0 (0)
C578L/10	*In vitro*	110	2 (1.8)	1 (0.9)	3 (2.7)
	In vivo	47	0 (0)	0 (0)	0 (0)
TO	*In vitro*	160	5 (3.1)	2 (1.3)	7 (4.3)
	In vivo	215	1 (0.5)	2 (0.9)	3 (1.4)
(C5BL/10 × CBA) F_1	*In vitro*	166	2 (1.2)	2 (1.2)	4 (2.4)
	In vivo	205	2 (1.0)	1 (0.5)	3 (1.5)

[a]From Maudlin and Fraser (*13*).

FIGURE 3. Trisomic first-cleavage mouse embryo; sperm donor was a TO male and thus there is no marker chromosome. The female-derived complement has 21 chromosomes.

females, and this was due to an increase in the incidence of $n+1$ egg-derived, rather than sperm-derived, complements [(Table 4) (*14*)].

Discussion

The methods detailed above provide rapid, accurate and efficient analysis of chromosomes in the first-cleavage mouse embryo. Large numbers of eggs are obtained when ovulation is stimulated with exogenous hormones (mean of 20-40/mouse) (*2*), and we have routinely processed up to 200 eggs/per day with one person making the preparations. Given the high proportion of preparations which are suitable for analysis, it is clear that a great deal of information can be obtained in a relatively short period of time. Because of the differential condensation of maternal and paternal chromosome complements, the source of aneuploidy can be accurately assigned; indeed,

Table 3. Synchrony of fertilization in an *in vitro* system.[a]

Sperm pre-incubation time, hr	Fertilized	Anaphase	Telophase-second polar body	Full sperm head decondensation
0.5	79/91 (87%)	67/79 (85%)	12/79 (15%)	3%
2	68/75 (91%)	16/68 (24%)	52/68 (76%)	61%

[a]Sperm suspensions were preincubated for the time noted, diluted and eggs were added. After 1 hr 15 min, eggs were fixed in buffered formalin, stained and assessed for fertilization.

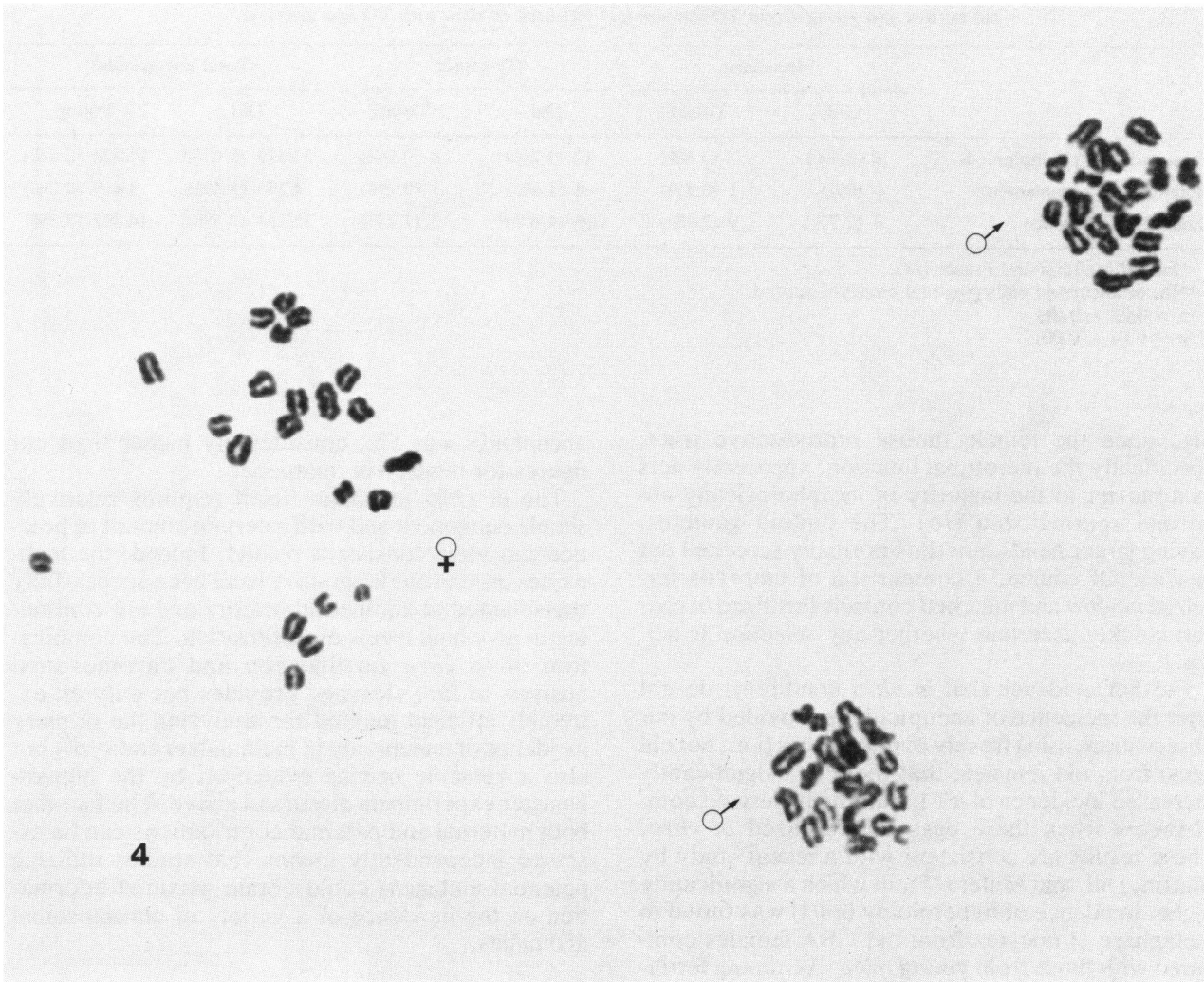

FIGURE 4. Tetrasomic dispermic triploid first-cleavage mouse embryo. The two sets of male-derived chromosomes have 20 each, the female-derived complement, 22. From Maudlin and Fraser (*13*), with permission.

either or both parent could be treated with potential mutagens and the incidence of anomalies from each source determined independently.

In the methods described here, two procedures might be objected to for possible effects on aneuploidy, namely superovulation and fertilization *in vitro*. While most of our studies have used both *in vivo* and *in vitro* fertilization and have detected no differences in aneuploidy, all eggs were obtained by superovulation and so any effect on nondisjunction should be present in both groups. A recent study by Takagi and Sasaki (*15*) has specifically examined this possibility and their data reveal no significant differences between eggs obtained by superovulation and spontaneous ovulation in the incidence of aneuploidy.

With respect to fertilization *in vitro*, our results indicate that the system itself does not generate aneuploidy, there being no significant differences between embryos fertilized *in vivo* and *in vitro*. There is no evidence either that the completion of meiosis II by the oocyte in an *in vitro* environment has any effect on nondisjunction or that spermatozoa with abnormal chromosome numbers are selected for or against *in vitro*, at least with respect to $n+1$ and $n-1$ complements. The one notable exception lies with spermatozoa possessing a diploid complement. Here there is a definite advantage *in vitro* for such gametes, with about 1% of all fertilization *in vitro* being accomplished by diploid spermatozoa while only 1 has been found in all of the *in vivo* fertilized embryos (> 2000) examined. This is not too surpris-

Table 4. Incidence of aneuploidy in first-cleavage mouse embryos derived from the eggs of old parous and young virgin TO females and fertilized *in vitro* with TO spermatozoa.[a]

	Monsomic		Trisomic		Total aneuploids[b]	
	Old	Young	Old	Young	Old	Young
Female-derived complement	9 (2.4%)	7 (1.6%)	12 (3.2%)[c]	4 (0.9%)	21/375 (5.6%)[c]	11/434 (2.5%)
Male-derived complement	0 (0%)	2 (0.5%)	4 (1.4%)	1 (0.2%)	4/293 (1.4%)	3/419 (0.7%)
Embryonic incidence	9 (2.7%)	9 (2.1%)	16 (4.8%)[d]	5 (1.2%)	25/334 (7.5%)[c]	14/427 (3.3%)

[a]Data of Maudlin and Fraser (*14*).
[b]No. of aneuploid embryos/total embryos scored.
[c]$p = 0.05 - 0.01$.
[d]$p = 0.01 - 0.001$.

ing, since the female mouse reproductive tract, specifically the uterotubal junction, apparently acts as a barrier to the majority of morphologically abnormal spermatozoa (*16*). The diploid gametes, having larger heads, are thus normally screened out *in vivo*. Of course, a comparison of embryos fertilized *in vitro* and matched controls fertilized *in vivo* can quickly ascertain whether any selection is acting.

Further evidence that *in vitro* conditions do not alter the incidence of aneuploidy is provided by our observation, using freshly ovulated eggs (i.e., not old eggs) from old females, that there is a significantly increased incidence of $n+1$ maternally derived complements when these eggs are fertilized *in vitro*. These results are consistent with a recent study by Martin, Dill, and Miller (*17*), in which a significantly higher incidence of hyperploidy ($n+1$) was found in metaphase II oocytes from old CBA females compared with those from young mice. Assuming fertilization by haploid spermatozoa and normal completion of meiosis II, such oocytes would give rise to the trisomic embryos detected in our study.

While it is obvious that our methods are most easily applied to studies utilizing the mouse, these basic techniques have recently been used to analyze human sperm chromosomes after fertilization, not of human eggs, but of hamster eggs (*18*). Briefly, since mature human metaphase II oocytes are difficult to obtain, hamster eggs have been used to test the fertilizing ability of human spermatozoa. To overcome the species specificity barrier, the zonae pellucidae are removed from hamster eggs and the human sperm suspensions then added. Having successfully fused with an egg, at least some spermatozoa can respond to the signals within the egg and undergo pronuclear formation; with the addition of a mitotic inhibitor, the chromosomes of the spermatozoa can then be analyzed. Thus far only 60 spermatozoa have been analyzed in this manner and the incidence of aneuploidy was 5%, considerably higher than our figures for mouse spermatozoa.

The *in vitro* technique itself requires relatively simple equipment and with a certain amount of practice can yield consistent results. Indeed, the techniques used in our laboratory have been successfully transplanted to another laboratory and are continuing to give high levels of fertilization. The combination of *in vitro* fertilization and chromosomal analysis at first cleavage provides not only an extremely efficient method for analyzing the primary incidence of aneuploidy in mammalian embryos, but also a versatile one as evidenced by the human-hamster experiments discussed above. The fact that both maternal and paternal contributions can be assessed independently means that studies utilizing potential mutagens could obtain maximal information on the incidence of a variety of chromosomal anomalies.

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